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A simple and rapid GC/MS method for the simultaneous determination of gaseous metabolites

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ABSTRACT

We modified and tuned a commercial model of a gas chromatography/mass spectrometry (GC/MS) instrument to develop a simple and rapid method for the simultaneous quantification of a variety of gas species. Using the developed method with the newly modified instrument, gas species such as H_2, N_2, O_2, CO, NO, CH_4, CO_2, and N_2O, which are common components of microbial metabolism, were accurately identified based on their retention times and/or mass-to-charge ratios (m/z) in less than 2.5 min. By examining the sensitivities and dynamic ranges for the detection of H_2, N_2, O_2, CH_4, CO_2, and N_2O, it was demonstrated that the method developed in this study was sufficient for accurately monitoring the production and the consumption of these gaseous species during microbial metabolism. The utility of the new method was demonstrated by a denitrification study with *Pseudomonas aureofaciens* ATCC 13985^T. This method will be suitable for a variety of applications requiring the identification of gaseous metabolites in microorganisms, microbial communities, and natural ecosystems.

1. Introduction

Gaseous compounds such as H_2, N_2, O_2, CO, NO, CH_4, CO_2, and N_2O are commonly produced and/or consumed by microorganisms during normal metabolic processes (Hughes, 1985; Conrad, 1996). Since the microbial production and consumption of these gaseous compounds via a variety of reductive and oxidative processes are interconnected, the development of a method for the simultaneous quantification of multiple gas species is highly relevant to the studies of microbial physiology and ecology.

To date, these common gas species have been quantified mainly by gas chromatography (GC) in combination with a number of detection techniques (reviewed by Crill et al., 1995), with individual or related groups of gas species requiring separate detection with specific detectors. For example, H_2, N_2, O_2, and CO_2 have been typically detected using a thermal conductivity detector, N_2O by an electron capture detector, and CO and CH_4 using a flame ionization detector. To overcome this inconvenience and facilitate more rapid sample processing, techniques for the simultaneous detection of multiple biogenic gas species using a single apparatus have been developed. For example, a GC system equipped with multiple columns and detectors with flow switching valves has been constructed (Hedley et al., 2006; Sitaula et al., 1992; Wang and Wang, 2003; Yoh et al., 1998); however, such GC systems consist of a number of components and require high technical skill to operate the complex assembly for redirecting the gas flow into the associated chromatographic systems.

Presently, GC-mass spectrometry (GC/MS) is used largely for the identification of microbial metabolites such as amino acids, sugars, and organic acids (Koek et al., 2006; Strelkov et al., 2004; Tian et al., 2009), but has also been applied for the detection some gaseous metabolites produced by microbes (Amano et al., 2008, 2011; Bazylinski et al., 1986; DeRito et al., 2005; Garber and Hollocher, 1982; Goretski and Hollocher, 1990; Liou et al., 2008; Liu et al., 2006; Shoun and Tanimoto, 1991; Waki et al., 2010). With the advent of GC/MS technology, particularly its enhanced sensitivity, the applicability of GC/MS has expanded to include the quantification of environmental ambient gasses existing in extremely low concentrations, such as CH_4, CO_2, and N_2O (Ekeberg et al., 2004). One of the advantages of GC/MS over GC is that it can be applied in stable isotope tracer experiments to evaluate metabolic fluxes in cellular and ecosystem processes.

Despite the high potential utility of GC/MS in gas analyses, the configuration of GC/MS systems has not yet been optimized for the rapid and broad-dynamic-range quantification of gaseous microbial metabolites. In addition, it has not been optimized for determination of wide variety of gas species. Considering that multiple gas species at various concentration levels are produced and consumed by microbes and microbial communities, the importance of the modification and optimization for these purposes are highly obvious. It would be

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noteworthy that although such gas species as N_2 and O_2 are major substrates/metabolites for microbes, it is difficult to obtain accurate and reproducible results when attempting to quantify N_2 and O_2 that are present in samples at as low level as 100 ppm. This would be largely because contamination of such gas species from ambient air invariably occurs when gaseous samples are injected through an injection port. Thus, preventing air contamination during sample injection is the key technique to achieve the purposes.

Here, we first made an attempt to prevent air contamination by modifying the injection port of a commercially available GC-quadrupole MS instrument. Second, we demonstrated the sensitivity and dynamic range of several gas species commonly found in microbial gas metabolism with an optimized analytical conditions which would enable the rapid and simultaneous quantification of a variety of gas species ranging from the level of ppm to percent (in volume) using a single GC/MS apparatus. We then examined the usefulness of the new method and modified instrument during a denitrification experiment with *Pseudomonas aureofaciens* ATCC 13985^T.

2. Materials and methods

2.1. Instrumentation and operating conditions

Fig. 1 shows a scheme of the quadrupole GC/MS system (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) equipped with a CP-PoraPLOT Q-HT column (25 m × 0.32 mm; Varian, Inc., CA, USA) used in this study. Several modifications were made to the commercial GC/MS model to minimize the contamination of ambient air into the analytical samples. The injection port (EN2SI, ZF2SI, and SI4G, Valco Instruments Co. Inc., Houston, TX) and the 8-port valve (2C8WE-PH, Valco Instruments Co. Inc.) were covered by a jacket through which ultrapure helium (He, >99.99995%) was flowing constantly at 40 ml/min. For measurements, a 200-μl gas sample was injected using a gas-tight syringe (PS-050033, Valco Instruments Co. Inc.) via the injection port into either a 25- or 50-μl sample loop (AL25CW and SL50CW, respectively, Valco Instruments Co. Inc.) that were fitted to the 8-port valve. Once the sample loop was filled with an injected sample, the valve was switched manually to supply the gas sample to the GC separation column. By using the 8-port valve fitted with two sample loops, precise injections with two different amounts (25 and 50 μl) could be accomplished. Between sample injections, the injection port and the two sampling loops were flushed with ultrapure He. Sampling needles and syringes were also flushed with a stream of ultrapure He immediately prior to each sampling. A coiled stainless steel tube (50 cm × 1 mm in diameter) was attached to the split gas vent port and the purging gas vent port for septum injection to prevent the backflow contamination of ambient air (Fig. 1).

For GC separation, a 200-μl sample was injected via the sample loop into the separation column, which was heated at 50 °C, with a split ratio of 30. Ultrapure He at a flow rate of 2.0 ml min^{-1} was used as a carrier gas. For the MS detection after the electron-impact ionization, the detection voltage was set at 0.8 kV. Mass spectra were obtained in the selected ion monitoring (SIM) mode to ascertain higher sensitivity and selectivity. Other detailed conditions of gas sample injection, GC separation and MS detection are shown in Table 1.

2.2. Preparation of standard gas samples

To generate standard curves for the GC/MS analysis, pure O_2, N_2, H_2, CH_4 and CO_2 (>99.9%; GL Sciences, Inc. Tokyo, Japan) and N_2O (>99.5%; GL Sciences, Inc.) were diluted in ultrapure He from ppm (10^{-6} vol./vol.) to percent (10^{-2} vol./vol.) orders as follows. First, the volume of each rubber-stoppered bottle was precisely determined from the difference between the water-filled and empty weights. Each bottle was then filled with either He or N_2 up to approximately 1.5 atm with a gas pressure injector (Sanshin Industrial Co. Ltd., Kanagawa, Japan), and a known amount of standard pure gas was injected into the bottle using a gas-tight syringe. The pressure in the bottle was measured with a manometer (Nidec Copal Electronics Co., Tokyo, Japan) for the precise calculation of gas concentrations. The diluted gas samples thus prepared were stored at least overnight with stirring using a magnetic bar to attain complete homogenization prior to use. Gas injections using a gas-tight syringe and pressure measurements were conducted in an ultrapure He stream.

To ascertain the detection limits and dynamic ranges of the analysis, 8 or 9 sequential concentrations in the following range of each gas species were prepared: 1.08×10^3 to 1.00×10^6 ppm for H_2, 5.41×10^2 to 2.06×10^5 ppm for N_2, 5.41×10^2 to 2.17×10^5 ppm for O_2, 7.20 to 2.17×10^5 ppm for CH_4, 7.20 to 2.17×10^5 ppm for CO_2 and 7.20 to 2.14×10^5 ppm for N_2O. To see if NO and CO can be clearly identified, CO

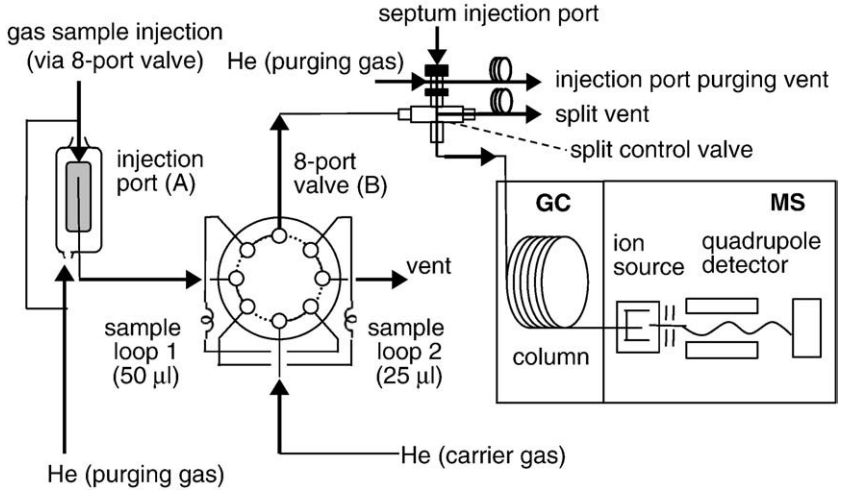

Fig. 1. Schematic diagram of the commercial quadrupole GC/MS analytical system modified in this study. Gas samples were injected using a gas-tight syringe via the injection port (A) into one of the two sample loops fitted to the 8-port valve (B). The injection port and the 8-port valve were enveloped by He flowing in the surrounding jacket to avoid contamination with ambient air. Once the sample loop was filled with the injected sample, the valve was switched and the gas sample present in either of the sample loops was subsequently loaded on the streamline and conveyed by the carrier gas stream into the GC separation column. The gas components of the sample were separated on the separation column and each gas species was detected by the specific mass-to-charge ratio in the quadrupole detector.

Table 1
Operational conditions of GC/MS.

Injection	Split ratio	30
	Injection volume	200 μl
	Temperature	100 °C
GC separation	Total flow	61.3 ml/min
	Head pressure	38.0 kPa
	Column flow	2.03 ml/min
	Linear velocity	56.6 cm/s
	Temperature program:	
	50 °C (2.5 min) —100 °C/min→ 110 °C(0.2 min)	
MS detection	Ionization energy	70 eV
	Ion source temperature	200 °C
	Interface temperature	250 °C
	Detection voltage	0.80 kV
	Analytical mode	SIM

and NO labeled with a stable isotope (^{13}CO and ^{15}NO, SI Sciense Co., Ltd., Saitama, Japan) were used. To investigate the effects of two background gasses, ultrapure He (>99.99995%) and ultrapure N_2 (>99.9995%), on the sensitivity of the detection of O_2, a dilution series of O_2 in He and N_2 matrices (1.25%, 2.46%, 4.82%, 9.35%, 17.2%, 20.7%, and 23.9% in the He matrix and 1.23%, 2.43%, 4.76%, 9.30%, 21.4%, and 25.6% in the N_2 matrix) were also prepared.

2.3. Monitoring of gaseous metabolites in a culture of a denitrifying bacterium

The denitrifying bacterium *Pseudomonas chlororaphis* subsp. *aureofaciens* ATCC 13985^T, which produces N_2O as the end product of denitrification (Casciotti et al., 2002; Firestone et al., 1979; Matsubara and Zumft, 1982; Sigman et al., 2001), was used in this study. The strain was grown in a batch culture overnight at 23 °C in 80 ml of Tryptic Soy Broth (Difco Laboratories) amended with 10 mM KNO_3, 1 mM NH_4Cl, and 36 mM KH_2PO_4 contained in a 120-ml butyl rubber-stoppered serum bottle. After an overnight incubation at 23 °C with mixing on a magnetic stirrer, a portion of the culture was transferred to a serum bottle containing 80 ml of the fresh medium to give a turbidity of $A_{600} = 0.01$. Air was then added to the headspace of the bottle using a syringe until the pressure reached 1.5 atm. The subculture thus prepared was cultivated for 48 h at 23 °C with mixing on a magnetic stirrer. Bacterial growth was monitored by measuring the turbidity of the culture at A_{600} with a spectrophotometer. Every 4 h, 200 μl of headspace gas was sampled and injected into the GC/MS to determine the O_2, CO_2, and N_2O concentrations by the method described earlier.

3. Results and discussion

3.1. Simultaneous detection of nine gas species

A mixture of H_2, N_2, O_2, Ar, ^{13}CO, ^{15}NO, CH_4, CO_2, and N_2O was prepared in a He matrix, and each of the nine gas species was identified using the modified GC/MS instrument by the combination of retention time and mass-to-charge ratios (m/z) (Fig. 2). The mass chromatogram of the mixture of the nine gas species in the He matrix is presented in Fig. 2a–i in the order: H_2, N_2, O_2, Ar, ^{13}CO, ^{15}NO, CH_4, CO_2, and N_2O. The stable isotope labeled compounds, ^{13}CO (m/z 29) and ^{15}NO (m/z 31), which can be identified based on the unique m/z of molecular and/or fragment peaks, were used for identifying the gas species unambiguously. Although three gas species N_2, CO and NO were separated based on their retention times with the analytical condition employed here (Fig. 2), we used ^{13}CO and ^{15}NO to make sure of the clear separation of CO and NO from peaks with m/z 28 and 30 derived from different molecules, such as ^{14}N–^{14}N and ^{15}N–^{15}N of

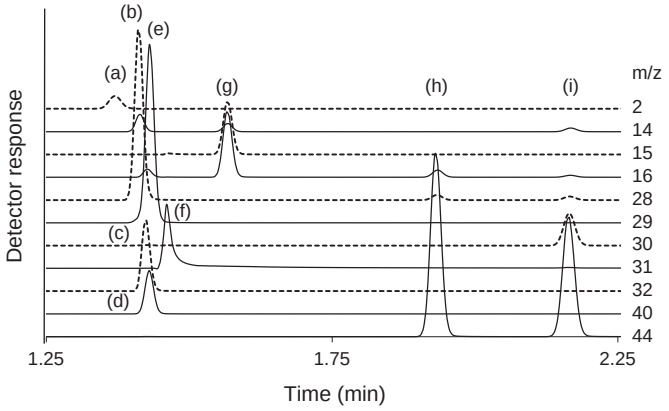

Fig. 2. The mass chromatogram of a mixture of nine gas species; H_2 (a, m/z 2), N_2 (b, m/z 14, 28), O_2 (c, m/z 16, 32), Ar (d, m/z 40), ^{13}CO (e, m/z 29), ^{15}NO (f, m/z 31), CH_4 (g, m/z 15, 16), CO_2 (h, m/z 28, 44), and N_2O (i, m/z 30, 44) in a He matrix.

N_2. As shown in Fig. 2, all gas species in the mixture were successfully separated with respect to their retention times and/or m/z values of molecular and fragment peaks; however, a few of the gas species, such as N_2, O_2, CO, NO and CH_4 possibly overlap their retention times or fragment peaks, especially when a particular gas species is abundant. In such instances, the examination of several molecular and fragment peaks is necessary for the accurate identification of the peak. For example, if a peak of the molecular ion of CH_4, CH_4^+ (m/z 16) overlaps with a fragment ion peak derived from O_2, O^+ (m/z 16), a fragment ion of CH_4, CH_3^+, m/z 15, can be selected for identification of CH_4. Alternatively, clearer separation of gas chromatographic peaks could be attained by lowering the flow rate of carrier gas. It is also a useful option to employ a stable isotope labeled compound as a substrate, which sometime result in providing a molecular/fragment peak with a unique m/z value, for monitoring microbial metabolites.

Using this analytical approach, the simultaneous identification of gas species commonly found in microbial cultures was possible. The total time for analyzing a single sample was 2.5 min (Fig. 2), which represents a more rapid, or similar, time than those of previously described GC systems (Hedley et al., 2006; Sitaula et al., 1992; Wang and Wang, 2003; Yoh et al., 1998).

3.2. Detection limits and dynamic ranges of the analysis

We estimated the limit of detection (LOD) of H_2, N_2, O_2, CH_4, CO_2, and N_2O using the IUPAC criteria (Long and Winefordner, 1983):

$$\mathrm{LOD} = k \times sd / m$$

where k is a numerical factor to determine the confidence level ($k = 3$ was used to provide a confidence level of 90% or greater), sd is the standard deviation of the responses, and m is the slope of the linear regression line obtained with the data of the three lowest concentrations of each standard gas, except for m/z 28 of CO_2 (57.3, 287, and 529 ppm) because the peak of 7.20 ppm was too small to quantify. The linear regression equation and the estimated LOD for each gas species are summarized in Table 2. The relative intensities of each fragment ion peak to the corresponding molecular ion peak were calculated by the slope of the standard curve of the fragment ion divided by that of the molecular ion, and these values are also shown in Table 2. As ambient air was an unavoidable contaminant during the preparation of the dilution series of gas standards and the sampling and injection of the samples, positive values of the y-intercept were obtained for gas species abundantly present in air. In contrast, the negative values of the y-intercept of gas species that were less abundantly present in air could be explained by systematic errors in preparing gas standards.

Table 2
The linear regression equations, estimated limits of detection (LODs) and relative intensity of fragment ion. The three lowest concentrations quantified were used to obtain linear regressions ($n = 10$ for each concentration). Correlation coefficient, R^2, of all equations was approximated to be 1.00.

Gas species	Mass to charge	Ion species	Equation for standard curve	LOD (ppm)	Relative intensity of fragment ion (%)
H_2	2	H_2^+	$y = 0.2x - 34.0$	335.9	100
N_2	28	N_2^+	$y = 49.1x + 4469.0$	60.5	100
	14	N^+	$y = 1.8x - 32.0$	81.8	3.7
O_2	32	O_2^+	$y = 36.9x + 1522.7$	69.6	100
	16	O^+	$y = 1.4x + 14.1$	79.6	3.8
CH_4	16	CH_4^+	$y = 21.2x + 3.1$	3.3	100
	15	CH_3^+	$y = 17.4x + 3.3$	3.8	82.1
CO_2	44	CO_2^+	$y = 74.6x + 61.3$	5.1	100
	28	CO^+	$y = 6.2x + 93.7$	29.2	8.3
N_2O	44	N_2O^+	$y = 52.8x - 17.6$	2.2	100
	30	NO^+	$y = 14.0x - 0.4$	5.5	26.5

The standard curves generated for all of the gas species were linear up to 20% in volume for O_2, CO_2, N_2, N_2O, and CH_4, and to 100% for H_2 (Table 2, Fig. 3). According to IUPAC (1997), the dynamic range is defined as a ratio between the maximum concentration and LOD for each gas species where a linear relationship between the gas concentration and peak area was maintained. The calculated dynamic range for each gas species was: 3.0×10^3 for H_2, 3.4×10^3 and 2.5×10^3 for m/z 28 and 14 of N_2, 3.0×10^3 and 2.6×10^3 for m/z 32 and 16 of O_2, 6.6×10^4 and 5.7×10^4 for m/z 16 and 15 of CH_4, 7.2×10^4 and 2.8×10^4 for m/z 44 and 28 of CO_2 and 9.7×10^4 and 3.9×10^4 for m/z 44 and 30 of N_2O. In this study, the detection voltage and the split ratio determining the injected sample volume were optimized to simultaneously quantify CH_4, CO_2, and N_2O in the range of less than 5 ppm and up to 20%. If necessary, the detection limit could be lowered using a higher detection voltage and a lower split ratio. In fact, N_2O in air (310 ppb) was clearly detected with a detection voltage of 1.2 kV and a split ratio of 10 using the 25-μl sample loop (data not shown). Conversely, the split ratio could be increased to quantify gasses present at higher concentrations.

The sensitivity for H_2 was lower than that observed for the other gas species likely due to the high fragmentation efficiency of H_2 to H^+ (m/z 1), which was undetectable by the GC/MS setup used in this study (Table 2). However, the lower sensitivity for H_2 may not affect the utility of GC/MS as the H_2 concentrations commonly

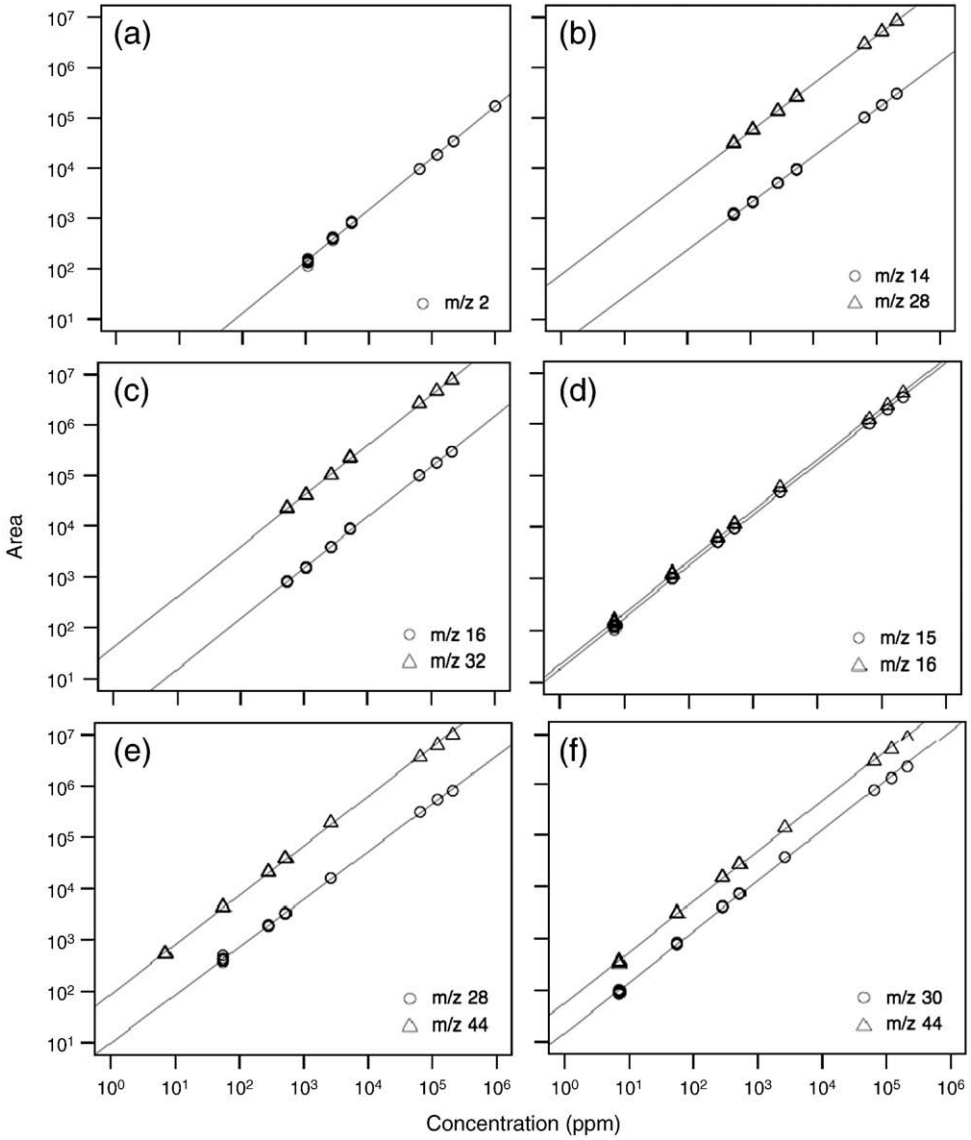

Fig. 3. Standard curves on a double logarithmic scale for the estimation of dynamic ranges for H_2 (a, m/z 2), N_2 (b, m/z 14, 28), O_2 (c, m/z 16, 32), CH_4 (d, m/z 15, 16), CO_2 (e, m/z 28, 44), and N_2O (f, m/z 30, 44) in a He matrix. The square of the sample correlation coefficient, R^2, of all the regression lines on the double logarithmic scale were above 0.999. Ten replicate injections for the three lowest concentrations, and four replicate injections for the higher concentrations were carried out.

encountered in microbial samples (Aguiar et al., 2004; Boga et al., 2007; Cadillo-Quiroz et al., 2008; Hetzer et al., 2008) are higher than the LOD for H_2.

3.3. Effects of background gasses on the detection sensitivity for various gasses

To investigate the effect of background gas species on the gas detection sensitivity, sensitivities for O_2 detection in a N_2 and He matrix were examined. As shown in Fig. 4, a much larger peak for O_2 in the He matrix was observed compared to the corresponding peak in the N_2 matrix. Since the retention time of N_2 is similar to that of O_2, it is possible that O_2 molecules co-migrate with the abundant N_2 molecules in the capillary column, and subsequently enter the ionization chamber together. Consequently, the ionization of O_2 may be suppressed by the presence of high concentrations of N_2. Thus, when a background gas of the sample is composed mainly of N_2, such as that found in air, standard gas samples should be prepared in a N_2 matrix for the quantification of gas species, such as O_2 and CO, that have retention times that may overlap with that of N_2. If the background gas is different from the carrier gas, as the background gas is typically much more abundant than other gas species, tailing of the background gas peak may result in an overlapping effect that is irrespective of gas species. Thus, the standard gas should be prepared in the identical gas species as that of the background gas of the sample.

3.4. Monitoring gaseous metabolites in a denitrifying bacterium

To examine the utility of our method, we monitored changes in the gas composition of O_2, CO_2, and N_2O in the headspace of a culture of the denitrifying bacterium *P. aureofaciens* ATCC 13985^T for 48 h (Fig. 5). The growth and denitrification activity, as determined by N_2O production of *P. aureofaciens*, could be distinguished into three phases: (1) an aerobic growth phase, (2) a temporary growth arrest, and (3) an anaerobic growth phase. This result clearly indicates that *P. aureofaciens* ATCC 13985^T performed O_2 respiration at the beginning of the cultivation, and subsequently switched to nitrate respiration (N_2O generating denitrification) as the concentration of O_2 in the headspace fell below 10%. Notably, the onset of nitrate respiration did not associate with the cell growth, but the temporary growth arrest was observed. Thus, the simultaneous measurement of gaseous substrates, gaseous metabolites, and bacterial growth enabled us to identify this interesting aspect of *P. aureofaciens* respiration, which will be the subject of future studies.

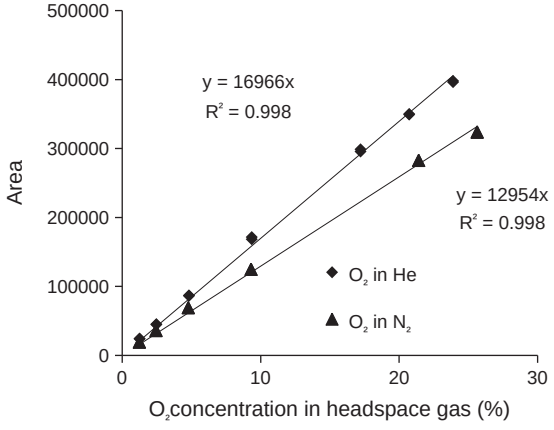

Fig. 4. Standard curves for the O_2 concentration in He and N_2 matrices. All data are shown as the mean of $n = 2$.

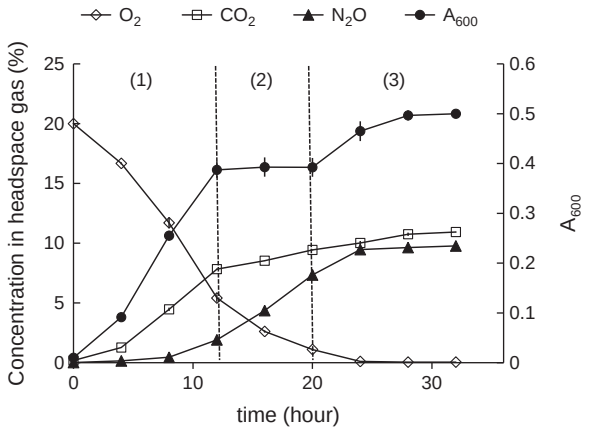

Fig. 5. Concentrations of O_2, CO_2, and N_2O in the headspace of a rubber-stoppered serum bottle containing a culture of *P. aureofaciens* in 10 mM nitrate-amended medium. The gas concentrations and A_{600} of the culture after a 48-h incubation period were 11.5 ± 0.0 % for CO_2, 10.1 ± 0.0 % for N_2O, 0.05 ± 0.01 % for O_2, and 0.51 ± 0.01 for A_{600}. Error bars represent the standard errors of means ($n = 3$), although some error bars are masked by the symbols.

To date, nearly all physiological studies on denitrifiers have been performed in either anaerobic or microaerobic conditions (Zumft, 1997), with only a limited number examining the effect of changes from aerobic to anaerobic conditions on denitrifying activities (Kester et al., 1997; Kornaros and Lyberatos, 1998; Molstad et al., 2007; Otte et al., 1996; Thomas et al., 1994) and gas production of other microorganisms (Kampschreur et al., 2008; Yu et al., 2010). The primary reason for the scarcity of such studies may be the lack of suitable and simple methods for the analysis. Aerobic/anaerobic conditions are one of the most critical factors determining microbial lifestyles and hence biogeochemical cycles (Brune et al., 2000; Megonigal et al., 2003). The significant improvement in gaseous metabolite analysis demonstrated in the present study is expected to facilitate future studies on microbial ecology and physiology.

4. Conclusions

Here, we established a novel GC/MS-based analytical method for the rapid, simultaneous quantification of most of the gaseous compounds found in microbial metabolism in a mixed gas sample. We showed that several gasses could be accurately quantified in a single analysis within 2.5 min (Fig. 2), with high sensitivity and a wide dynamic range (Table 2 and Fig. 3). It would be noteworthy that most abundant atmospheric gas species, N_2 and O_2, can be successfully determined as well (Figs. 2 and 3), by preventing air contamination occurring during sampling and sample injection processes, which was accomplished simply by covering the sampling and the injection ports with ultra pure helium stream (Fig. 1). The developed method can be applied not only to physiological studies in microbiology, as demonstrated here in a denitrification experiment with *P. aureofaciens* ATCC 13985^T, but also to other biological studies requiring the quantification of mixed gasses, such as gas metabolism in plants and breath tests for animals. By combining stable isotope tracer techniques, the applicability of the proposed method for microbiology could be greatly extended, providing a more detailed picture of metabolic pathways and kinetics of microbes and microbial communities (Amano et al., 2007, 2011; Katsuyama et al., 2008; Isobe et al., in press).

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